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DIETARY BASIS FOR DEVELOPMENTAL PLASTICITY OF AN ANDROCONIAL STRUCTURE IN THE SALT MARSH MOTH *ESTIGMENE ACREA* (DRURY)(LEPIDOPTERA: ARCTIIDAE).

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ABSTRACT. Larvae of the salt marsh moth *Estigmene acrea* (Dru.) feed on a variety of herbaceous plants including some that contain secondary plant substances called pyrrolizidine alkaloids (PAs). The salt marsh moth uses PAs in defense against predators and parasites and as pheromone precursors while non-PA plants are used for general nutrition. This study focuses on the development of their androconial organs, coremata, and on the role of pyrrolizidine alkaloids as morphogens stimulating corematal growth. During the pupal period, the development of the coremata can be divided into five discrete stages. Dietary PAs fed to laboratory raised final instar larvae were found to accelerate corematal development in stages four and five, to increase the number of corematal scales and to enhance corematal size compared to larvae without a PA dietary supplement. As part of the developmental process, dietary PAs stimulated the formation of a network of stellate cellular inclusions in the secretory cells at the base of each androconial scale. In addition, dietary PAs shortened the duration of the pupal stage for males but not females. The effects of PAs as morphogens in *E. acrea* are compared to those for the South Asian arctiines *Creatonotus gangis* and *C. transiens* in which the developmental role of PAs was first discovered.

Additional key words: coremata, hydroxydanaidal, morphogen, pyrrolizidine alkaloids

INTRODUCTION

Over twenty years ago the unusual mating systems of the arctiine moths *Creatonotus gangis* and *C. transiens* were described (Schneider *et al.*, 1982; Boppré and Schneider, 1985). In these South Asian insects males gather in aggregations and inflate impressive quadrifid abdominal coremata. Females are attracted to the aggregation by a pyrrolizidine alkaloid-derived pheromone hydroxydanaidal released by these structures (Wunderer *et al.*, 1986). Moreover, the size and development of the male's coremata are determined by larval access to pyrrolizidine alkaloids (Schneider *et al.*, 1982; Egelhaaf *et al.*, 1992). The mating systems of the *Creatonotus* species represent stunning examples of sex role reversal—males attracting females instead of the opposite (which normally occurs in moths)—the evolution of which is of considerable interest to behavioral ecologists. Little is known about the details of these interesting mating systems largely due to the difficulty in obtaining specimens and observing the behavior under natural conditions. It was recently discovered that the common New World species *Estigmene acrea*, also an arctiine, has many similarities to the *Creatonotus* species in behavior and ecology (Willis and Birch, 1982; Davenport and Conner, 2003; Jordan *et al.*, 2005). We herein describe the developmental events that give rise to the coremata in male *Estigmene acrea* and the effects of pyrrolizidine alkaloids on this developmental process. We compare our results to those previously reported for *Creatonotus* (Schneider *et al.*, 1982; Egelhaaf *et al.*, 1992).

The salt marsh moth, *Estigmene acrea*, hearafter referred to as *Estigmene*, has an unusual dual mating system (Willis and Birch, 1982). Early in the evening males gather in groups and inflate their abdominal coremata (Figure 1a). As in *Creatonotus gangis* and *C. transiens*, hearafter referred to as *Creatonotus*, females are attracted to the aggregation and mate. Later in the evening unmated *Estigmene* females revert to a more traditional lepidopteran mating scheme in which they release a blend of sex pheromones (Hill and Roelofs, 1981; del Mazo-Cancino *et al.*, 2004) and attract males. This alternative mating strategy was also noted in *Creatonotus*.

The larvae of the salt marsh moth are polyphagous and frequently include plants containing pyrrolizidine alkaloids (PAs) in their diet (Hartmann *et al.*, 2005). PAs are toxic secondary plant substances based on a bicyclic nitrogen-containing pyrrolizidine ring found sporadically in many plants, particularly in certain Asteraceae, Boraginaceae, and Fabaceae (Hartmann and Ober, 2000). Several arctiid species sequester them as defenses against predators and parasites (Weller *et al.* 1999; Hartmann and Ober 2000; Singer *et al.*, 2004; Hristov and Conner 2005). As in *Creatonotus*, larval *Estigmene* preserve the alkaloids through metamorphosis, and derivatives of the alkaloids serve as precursors for the male courtship (corematal) pheromone, hydroxydanaidal (Hartmann *et al.*, 2003; Hartmann *et al.*, 2004; Hartmann *et al.*, 2005; Jordan *et al.* 2005).

The coremata of male *Estigmene* consist of two inflatable tubes emanating ventrally from intersegmental membranes between abdominal segments seven and eight. The coremata are invested with elongate androconial scales that increase their surface area for the release of the pyrrolizidine alkaloid-derived pheromone hydroxydanaidal (Krasnoff and Roelofs, 1989; Jordan *et al.* 2005). The size of the

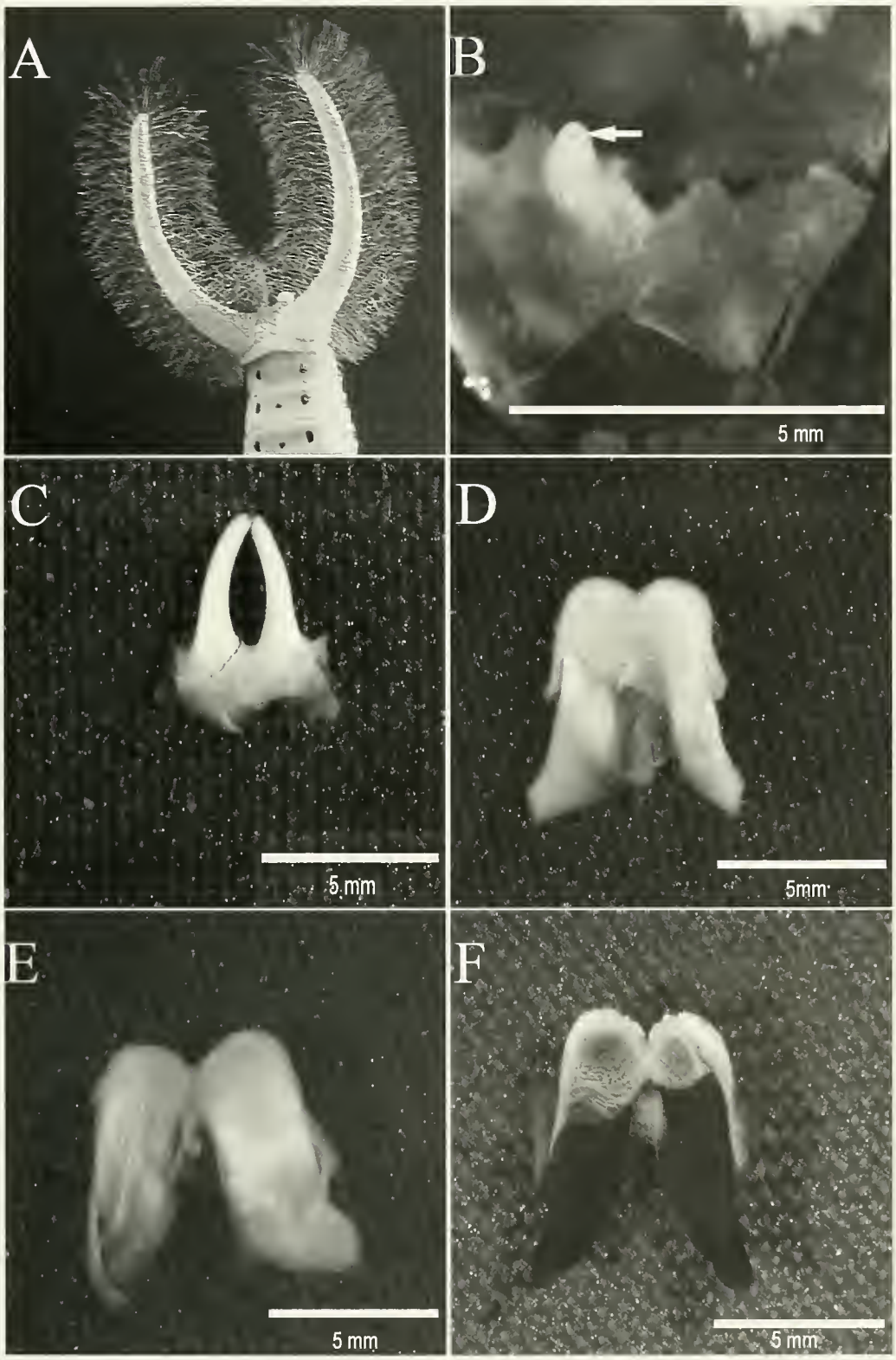

FIG. 1. (A) Artificially inflated coremata of adult male *Estigmene acrea*; (B-F) five different stages of coremata development; (B) stage one — the rapid proliferation of epidermal imaginal disks; (C) stage two—development of the imaginal disks into anteriorly oriented fingerlike projections; (D) stage three —the projections begin to regress and invaginate and form terminal buttons; (E) stage four— the corematal scales develop within a fine sheath (F) stage five—the corematal scales sclerotize and melanize, marking complete development.

coremata and their pheromone load in the adult male depend upon the quantity of pyrrolizidine alkaloids consumed in the larval stage (Davenport and Conner, 2003). Pyrrolizidine alkaloids thus have a specific morphogenetic effect stimulating coremata growth. We herein show how the PAs exert their effects on the development of the coremata of *Estigmene* and compare our results with those obtained earlier for *Creatonotus*. We propose *Estigmene acrea* as a readily accessable model for studying the behavior and evolution of sex role reversal in a pheromonal communication system.

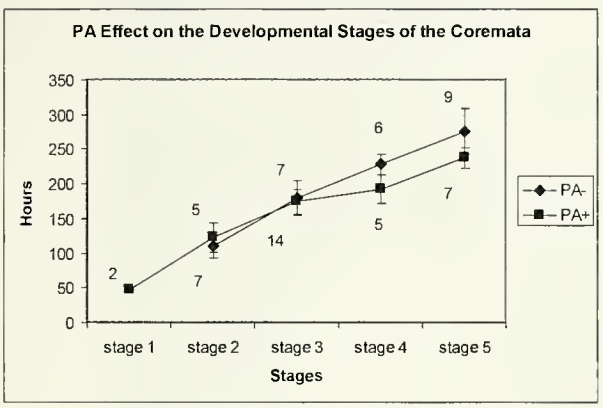

Fig. 2. Effect of dietary PAs on the rate of development of the coremata of *Estigmene acrea*. Symbols represent means, error bars represent standard deviations of the mean. Sample sizes are shown for PA+ males above the curve and for the PA- males below the curve.

METHODS

To determine the effects of dietary pyrrolizidine alkaloids on the development of coremata, we raised larvae of *Estigmene acrea* obtained from St. Charles Parrish, Louisiana, on a commercially available insect diet (salt marsh caterpillar diet, Bioserv # F9743B). Two hundred larvae were raised in disposable plastic petri dishes containing 1 to 5 cm^3 of diet (depending on larval size), replaced daily. Larvae were raised at room temperature (average 23°C). Larvae and pupae were exposed to a 16 hour light: 8 hour dark photoperiod. During the early hours of their final larval instar one-half of the larvae (designated PA+) were supplemented with 5 cm^3 of the diet containing 2.5 mg of the PA monocrotaline (Davenport and Conner, 2003). Once they had ingested all of the alkaloid diet, the PA+ larvae were returned to the normal diet feeding schedule. Controls animals (designated PA-) were offered an equal amount of PA-free diet. Monocrotaline was obtained from the Fairfield Chemical Company, Blythewood, South Carolina, and was certified 99% pure. Larvae were allowed to complete development to the pupal stage. After pupation, the pupae were then sexed, and the males and females were separated. Pupal tissues from four males were fixed in Bouin's fixative (Fisher Scientific) every 12 hours beginning with the onset of pupation and ending with eclosion. After a 48 hour fixation period, abdomens were rinsed and stored in 5ml of 70% ethanol. Abdomens were dissected in 70% ethanol and the development of the coremata assessed using light microscopy and documented by digital photography using an Olympus SZX-ILLK100 dissecting scope fitted with an Optronics digital camera. Developing coremata were assigned to five discrete stages of development (based on the findings of this study) and their age in hours noted. Thin sections of 20 (10 PA+ and 10 PA-) male pupae were prepared using a vibratome. The sections were stained using using a 1% Neutral Red solution in 0.1M acetate buffer for 1 minute. Stained sections were dehydrated using an ethanol series, followed by xylene. Sections were coverslipped using Permount (Fisher Scientific) and examined under a Zeiss Axioplan™ 40C microscope using normal light. Digital images were taken with a Hamamatsu© color chilled CCD camera.

The final number of androconial scales associated with single corema of 10 PA+ and 10 PA- males was assessed by snipping the scales from the uninflated corema and manually counting them. The scale counts were compared using a *t*-test (SPSS© 14.0 for Windows, SPSS Inc.).

RESULTS

The coremata of *Estigmene acrea* develop from epidermally derived imaginal discs as in *Creatonotus* (Egelhaaf *et al.*, 1992). Their growth and development are diet dependent. Visible growth of the disks begins approximately 48 hours after pupation and is complete just prior to adult eclosion.

The development of the coremata can be broken down into five discrete stages (Figure 1 b–f):

1. Epidermal imaginal disks associated with the intersegmental membrane between the seventh and eighth abdominal segments begin to proliferate rapidly. (Note this stage was never observed in PA- males probably because of the smaller size of the structures. Its presence is inferred from our observations in PA+ animals and the presence of the later stages in PA- animals.)

2. Imaginal disks develop into anteriorly oriented fingerlike projections.

3. Projections begin to regress, invaginate, and form terminal buttons.

4. Corematal scales develop within a fine acellular sheath.

5. Corematal scales separate, sclerotize, and melanize, marking complete development.

The timing of the initial stages of development of PA+ males were not distinguishable from those of control animals. However, PA+ males reached stages four and five more quickly than did the PA- males (Figure 2: Mann Whitney U test, $P<0.005$). PA+ males also developed more rapidly than PA- males, eclosing in 252 ± 2.5 hours (n = 10). PA- males eclosed in 324 hours ± 7.4 hours (n = 10), requiring an average of 3 additional days to complete pupal development. No difference was detected in the length of the pupal stage in females of the two categories. The resultant coremata of the PA+ males were roughly twice the size of those of PA- males (Figure 3) and had more scales (490 ± 30.8 [n=10] scales for PA+ males versus 197 ± 23.6 [n=10] for PA- males, Figure 4: *t*-test $p<0.001$). Since each scale has a socket and trichogen cell (secretory cell) at its base, the difference in the number of scales affected these components as well. The secretory cells associated with each scale are visible through the very thin cuticle of the coremata. This effect is heightened by the use of ultraviolet (360 nm) illumination (Figure 5).

Fig. 3. Coremata of *Estigmene acrea* at 100% development; (top) 2 PA- coremata from a male that received no monocrotaline in its final larval instar, and (bottom) corema from a PA+ male that received 2500 µg of monocrotaline in the final larval instar.

Thin sections revealed the intimate relationship between each scale, its cuticular socket, and an underlying trichogen cell (Figure 6a). A network of densely staining (neutral red) stellate cellular inclusions (5–10 microns in diameter) can be seen in chains traversing the secretory cells, the sockets and the lumen of the scent scales (Figure 6b). These inclusions were only seen in PA+ animals.

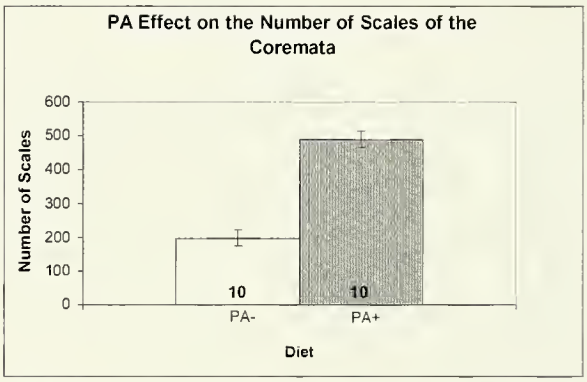

Fig. 4. Effect of dietary PAs on the number of corematal scales of Estigmene acrea. Mean values and standard deviations are plotted. (PA+ = 2500 µg monocrotaline). Sample sizes are indicated within each.

Discussion

As in *Creatonotus*, pyrrolizidine alkaloids play a vital role in sexual signaling of *Estigmene* by regulating the development of the androconial structures that release male courtship pheromones. PAs accomplish this by acting as morphogens triggering the accelerated growth and development of the coremata from their epidermal imaginal disks in the pupal stage. Under the influence of PAs, coremata grow more rapidly and reach a greater final size.

Our results are in many respects similar to those Egelhaaf *et al.* (1992) obtained for the arctiid *Creatonotos transiens*. PAs had a pronounced effect on the developmental trajectory of the coremata of both *Creatonotos* and *Estigmene*, although the effect was notably larger (10X) in the former. The developmental stages of the two groups are essentially identical except

FIG. 5. Fluorescent image of artificially inflated coremata of adult male *Estigmene acrea* that received monocrotaline in its last larval instar viewed under UV (360 nm.) illumination, arrows show individual secretory cells below each scale. .

that the coremata of *Creatonotos* are quadrifid. Egelhaaf *et al.* did not note cellular inclusions specific to PA+ animals that we have described. Since these inclusions were found only in PA+ males, we posit that they are involved in transport of the male courtship pheromone from its origin in the secretory cells to its release point.

Hartmann *et al.* (2004, 2005) showed that *Estigmene* larvae process ingested PAs in a series of steps. The PAs are first hydrolyzed to the simple necine base retronecine, which is re-esterified to form insect-specific alkaloids (eg. Creatonotine B). In males, these insect-specific alkaloids are used to produce the courtship pheromone, hydroxydanaidal. We do not yet know which of these alkaloidal materials stimulates corematal growth. It seems unlikely that it is hydroxydanaidal because this compound does not appear until well after the coremata are fully formed (Nickisch-Rosenegk, *et al.*, 1990, Hartmann *et al.*, 2004).

The strength of the morphogenetic effect of PAs, which has now been detected in *Creatonotos* and *Estigmene*, acts as a measure of the importance of PAs in their natural history and in the sexual system of each species. It should be noted that not all PA-feeding arctiids show the effect. Methods identical with those described herein did not detect this effect in *Pyrrharctia isabella* (E. McCammack and W. E. Conner, unpublished data). Krasnoff and Roelofs (1989) argued that the alkaloid-based pheromonal communication system of *P. isabella* has become vestigial in that species and McMammack and Conner's findings support their contention.

The pupal stage of male *Estigmene* exposed to PAs in their larval stage is significantly shortened. Egelhaaf *et al.* (1992) did not note an effect on the duration of the pupal stage in *Creatonotus*, but a similar effect was noted for larvae and adult females in the arctiid *Utetheisa ornatrix* (del Campo *et al.* 2005). For *Utetheisa* it was argued that the shortening of life stages

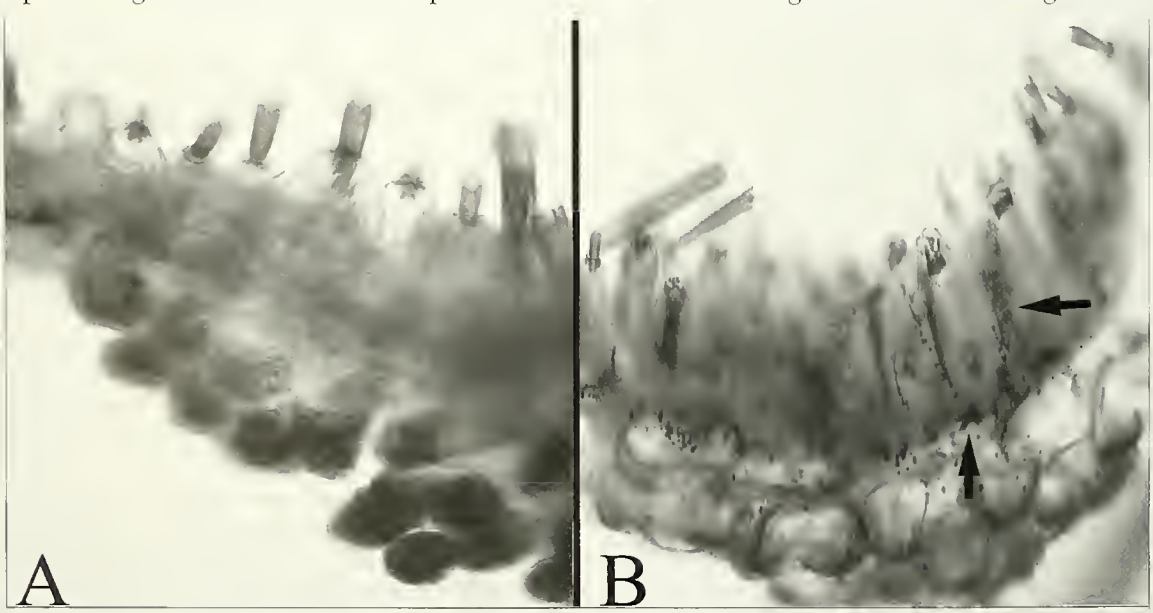

FIG. 6. Ultra thin cross sections of coremata: (A) section magnified at 20X from a male that received no PAs in its larval state (B) section magnified at 10X from a male that received 2500 µg of monocrotaline in its last larval instar, arrows highlight heavily stained stellate cellular inclusions

in the presence of PAs is a competitive strategy to gain a numerical advantage over slower growing PA-deprived individuals given a patchy host plant environment. The significance of pupal shortening in male *Estigmene* is unknown but it could conceivably be part of a similar adaptive strategy.

Overall the behavior, physiology, and development of *Estigmene* are similar to that of *Creatonotus*. The exact phylogenetic relationship of the two genera is not yet known, but they are likely to be closely related (Ferguson, 1985). It is possible that their reliance on PAs as morphogens and behavioral regulators stems from a common PA-feeding ancestor (Weller *et al.*, 1999). Because of the behavioral, developmental, and ecological similarities between *Creatonotus* and *Estigmene* we propose the latter as a model system that will provide insight into the behavior and evolution of the extraordinary sex role reversal behavior in both species.

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